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The inactivation of endotoxin by serum: A phylogenetic and ontogenetic study

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THE INACTIVATION OF ENDOTOXIN BY SERUM:

A PHYLOGENETIC AND ONTOGENETIC STUDY

By

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ABBREVIATIONS

ACD	acid-citrate-dextrose
C	degrees Centigrade
g	gram
g	gravity
i.p.	intraperitoneal
i.v.	intravenous
LD_{50}	50% lethal dose
M	molar
mg	milligram
ml	milliliter
N	normal
PBS	phosphate-buffered-saline
s.c.	subcutaneous
ug	microgram

CHAPTER I

INTRODUCTION

Incubation of bacterial endotoxin with normal human serum or plasma causes a reduction in or a complete loss of many characteristic host-reactive properties normally elicited in animals injected parenterially with endotoxin (30). The humoral mechanism responsible for the alteration of endotoxin has not been well defined, in that characterization of the factors responsible for inactivation of endotoxin has been attempted without success (42). The design of this study was directed toward further characterising these factors by studying their phylogenetic and ontogenetic distribution in serum from various classes of animals.

Identification of the humoral mechanism responsible for the alteration of endotoxin could have important clinical significance. Invasion of the blood stream with gram-negative bacteria may result in irreversible shock leading to death (55). Over the past twenty-one years the common occurrence of bacterial shock in humans has been recognized (60). In a recent study Myerowitz et al. (31) found 10.7 cases of gram-negative bacillemia per 1000 admissions to the Peter Bent Brigham Hospital in Boston occurring over a period of 19 months; 25.2% of these cases were fatal. Fine (14), in an accumulative review of several years of work, strongly implied that the irreversible shock associated with gram-negative septicemia is caused by the endotoxin component of these bacteria. Recently, Skarnes (51) reemphasized the importance of the humoral system responsible for endotoxin

alteration as a potential host defense mechanism. It seemed reasonable, therefore, that studies on the endotoxin altering system of blood should be continued; such information possibly could be used to design procedures which would enhance this naturally occurring system for therapy or prophylaxis.

At present, the major criterion for defining an endotoxin rests with the capacity of these substances to elicit characteristic host reactions. Milner et al. (30) have catalogued many of these reactions which include lethal shock, production of a biphasic fever, the Saranelli-Shwartzman reactions, hemmorraghic tumor necrosis, antigenicity for the production of homologous antibodies, and adjuvant activity.

Prolonged incubation of endotoxin with human serum results in the loss of many properties of endotoxin. Hegemann (18) first noticed that serum could reduce the pyrogenicity of endotoxin. Further work confirmed this reduction in the pyrogenicity of endotoxin after incubation with human serum and showed that the endotoxin altering mechanism was contained in Cohn Fraction IV_1 of human serum (58). The lethal effects of endotoxin also were altered by incubation of the endotoxin with human serum. Ho and Kass (19) observed that the lethal effect of endotoxin from Salmonella typhimurium injected into rats or embryonated chicken eggs was considerably reduced after incubation of the endotoxin with human serum or plasma. Reduction of the lethal effect of endotoxin of Salmonella typhosa for rabbits after incubation of the endotoxin with Cohn Fraction IV_1 of human plasma has been demonstrated by Rudbach and Johnson (46).

Tumor damage may also be induced by injection of endotoxin. This was reduced by first incubating the endotoxin with human plasma. For example,

Landy et al. (25) have shown that endotoxin injected into mice bearing Sarcoma 37 tumors would result in the hemmorraghic necrosis of 100% of the tumors. Incubation of the endotoxin with human serum before injection into the tumor bearing mice resulted in damage to only 20% of the tumors. Several of the immunologic properties of endotoxin were altered by incubation with human serum. As demonstrated by Stauch and Johnson (54), endotoxin in buffered saline was readily precipitated by homologous antiserum. After incubation of the endotoxin with normal human serum, less than 50% of the original amount of endotoxin was precipitated by homologous antiserum. Landy et al. (26) reported that endotoxin, normally antigenic in rabbits, lost much of its capacity to stimulate antibody production if incubated with human serum or plasma prior to injection. Cluff (7), using both the Oudin and Ouchterlony methods of immunodiffusion analysis, examined the precipitation patterns of endotoxin of Shigella flexneri. Endotoxin incubated in saline formed three distinct bands of precipitate when reacted with homologous antiserum. Incubation of the endotoxin with human serum resulted in the expresion of only one band upon immunodiffusion analysis. Rudbach and Johnson (44) were able to expand the findings of Cluff. As determined by double immunodiffusion patterns, they reported that incubation of endotoxin with Cohn Fraction IV_1 of human serum resulted in the loss of a slowly migrating antigen and the formation of a rapidly migrating antigen. This latter material in immunodiffusion plates gave a band of precipitate which formed a line of identity with the band of precipitate given by a small molecular weight hapten produced by acetic acid hydrolysis of endotoxin.

The observation of Hegemann (18) that serum could alter the endotoxin molecule, causing a substantial reduction in pyrogenicity, stimulated experimentation on this humoral system. Several theories have been postulated about the nature of the mechanism of inactivation of endotoxin. Keene et al. (23) varied experimental conditions and determined several factors which influenced the ability of human plasma to reduce the pyrogenicity of endotoxin from Serratia marcescens. The temperature of incubation, the relative concentrations of endotoxon and plasma, and the pH of the reaction mixture were all found to influence the rate of the reaction. Results obtained from these experiments were interpreted to indicate that the degradation of endotoxin by human plasma was an enzyme-catalyzed reaction. Although Keene et al. (23) did not identify the circulating enzyme responsible for the degradation of endotoxin, they postulated that such an enzyme might have been introduced into the plasma from the several tissues which had been shown to detoxify endotoxin.

Skarnes (49) reported that endotoxin, when exposed in vitro to human serum, complexed with alpha-1-lipoprotein and alpha-1-globulin. Both of these proteins were also shown to possess a nonspecific esterase activity. In vivo studies indicated that endotoxin interacted with a lipoprotein having esterase activity (50).

Experiments with cold ethyl alcohol fractions III_o and IV_1 of plasma have confirmed the fact that the endotoxin altering mechanism was contained therein (44, 46, 58). Both of these fractions contained lipoprotein (34). However, partial removal or dissociation of lipoproteins in normal human plasma or Fractions III_o and IV_1 did not change the ability of these substances to alter endotoxin (57). Yoshioka et al. (57)

a role of a serum alpha-globulin in endotoxin alteration. Yoshioka and Konna (59), by detailed fractionation of horse serum, isolated a serum alpha-2-macroglobulin capable of altering endotoxin.

The possibility that the endotoxin altering abilities of serum could be enhanced by immunization with endotoxin was examined by Jaques and Jaquet (20). Alteration of endotoxin with sera obtained from normal rats and rats immunized with endotoxin was assayed by recording the lethal effects of the endotoxin-serum incubation mixtures in adrenalectomized rats. Immune serum diluted 1:10 in saline had approximately the same capacity to alter endotoxin that undiluted normal serum possessed, with respect to rate of alteration and quantity of endotoxin altered. The greater ability of immune serum to alter endotoxin was attributed, in part, to the presence of antibodies specific for the endotoxin (19).

The possibility that components of the complement system were involved in the inactivation of endotoxin also has been considered. Rudbach (42) obtained evidence which suggested that C3 and C4 may be bound to endotoxin which has been incubated in human plasma. Beernink and Steward (5) found that C3, and possibly C4, were bound tightly to the surface of cells of E. coli after exposure of the bacteria to guinea pig serum. It has been reported that the sixth component of complement (C6) was necessary for the inactivation of endotoxin by rabbit serum (21).

Results obtained from treatment of endotoxin with detergents or other surfactants have given rise to a practical model for the inactivation of endotoxin by serum. Oroszlan and Mora (32) showed that endotoxin treated with sodium lauryl sulfate, a detergent, lost much of its capacity to cause necrosis of Sarcoma 37 tumors. Also, immunodiffusion

and ultracentrifugal analysis revealed the endotoxin was broken down into smaller subunits by treatment with sodium lauryl sulfate. It has been reported that treatment of endotoxin with this detergent also caused a dimunition in the capacity of the endotoxin to induce antibody production in rabbits (4). Ribi et al. (36) have found that macromolecular endotoxin was dissociated by the bile salt sodium deoxycholate into nontoxic subunits with molecular weights of about 20,000. Upon removal of the bile salt by dialysis, the subunits reaggregated into biologically active endotoxin. However, addition of a small amount of human plasma to the dissociated endotoxin prior to removal of the sodium deoxycholate prevented reassociation of the subunits and regaining of biological activity. The results of these and other experiments (37, 45, 47) led Rudbach et al. (43) to postulate a mechanism for alteration of endotoxin by serum. Endotoxin incubated with serum was dissociated by substances in the serum with surfactant properties. The smaller subunits were then bound by serum proteins and the endotoxin was thereby rendered biologically inactive.

The capacity to alter endotoxin has been demonstrated in plasma from animals other than the human. Keene et al. (23) examined the capacity of plasma from different species to inactivate endotoxin in vitro, and found that endotoxin incubated with sera from guinea pig, horse, dog, rabbit, cat, mouse, and rat lost much of its capacity to produce febrile responses in rabbits.

The chicken embryo has been found to be a useful model for examining the development of resistance to endotoxin. Smith and Thomas (53) observed that the susceptibility of chicken embryos to endotoxin decreased as the

embryo matured from day 10 to hatching. Endotoxins prepared from several gram-negative bacteria were inoculated onto the chorioallantoic membranes of embryonated eggs. Ten-day old embryos were highly susceptible to the lethal effects of endotoxins, while embryos 12-, 14- and 16-days old, were progressively more resistant to the lethal effects of the endotoxins. Finkelstein and Ramm (16) found that the chicken embryo became refractory to the lethal effects of intravenous endotoxin between the 11th and 15th day of incubation. There was a 10,000 fold increase in resistance to endotoxin in older embryos compared to the 11-day old embryo.

The alteration of endotoxin by incubation with human blood constituents and a variety of mammalian sera has been described above. This reaction may have some importance in protection of animals against the pathologic, including lethal, effects of bacterial endotoxemia (51). To reiterate, the chicken embryo becomes resistant to endotoxic lethality as a function of age. These two observations led to the following experimental design: (1) analyses of sera obtained from several animal species for their capacity to alter endotoxin; (2) the analysis of serum samples obtained from chicken embryos of varying ages for the capacity to inactivate endotoxin; (3) and relating the mechanism of alteration found in these various sera with that of human serum. This line of experimentation hopefully would lead to: (1) the discovery of sera which do and do not have an endotoxin altering system, on both a phylogenetic and ontogenetic scale, and the examination of such sera with the hope of further elucidating the nature of this system; and (2) the establishment of a correlation between the development of resistance in chicken embryos to the _in vivo_ administration of endotoxin and the capacity of serum from

chicken embryos of varying ages to inactivate the endotoxin *in vitro*. Finally, it is hoped that an endotoxin altering system similar to that in human serum can be found in the serum of a lower animal. The serum of such an animal might have a spectrum of plasma proteins less complex than that in human serum and this would ease the task of characterizing the system.

CHAPTER II

MATERIALS AND METHODS

Preparation of endotoxin from Escherichia coli 0113.

Endotoxin was extracted from Escherichia coli 0113 by modification of the aqueous-phenol method of Westphal et al. (61). The starting culture of E. coli 0113 (Braude strain) was supplied by Dr. K. C. Milner of the Rocky Mountain Laboratory, Hamilton, Montana. Preparation of endotoxin was accomplished in four general steps: Step 1, growing and harvesting the cells; Step 2, fractionation of cells and collection of cell walls; Step 3, extraction of endotoxin from the cell walls by aqueous-phenol method; and Step 4, precipitation of endotoxin with ethanol and sodium acetate, followed by lyophilization of the extracted endotoxin. The complete procedure is given in Figure 1. A total of 3.8 g of endotoxin was extracted from a wet weight of 254g of E. coli cells by this procedure. The endotoxin was designated as lot Ec 180.

Normal serum.

Normal sera from several species of animals was obtained as described in Table 1. For serum collected by the author, whole blood was allowed to clot at room temperature from 1 hour and at 4 C for 24 hours. The serum was separated from the clot by centifugation in an International Refrigerated Centrifuge, Model PR-6 (1000 X g for 30 minutes), decanted into vials, and frozen (-20 C) until needed.

Step 1.

Lyophilized preparation of Escherichia coli 0113 plated on nutrient agar and incubated for 18 hours at 37 C.

↓

Typical colony picked and inoculated into 100 ml of M-9 medium[1] and incubated at 37 C for 18 hours with shaking.

↓

Culture transferred three times to fresh M-9 and incubated at 37 C for 18 hours with shaking for each transfer.

↓

6 flasks, each containing 100 ml of M-9, inoculated following third transfer and incubated at 37 C for 18 hours with shaking.

↓

6 flasks, each containing 1 liter of M-9, inoculated with 1 flask of 100 ml of culture and incubated at 37 C for 24 hours with shaking.

↓

Each 1 liter of cells was used as inoculum for 10 liters of M-9 which was incubated at 37 C for 18 hours with sterile air bubbled through the medium.

↓

E. coli 0113 cells harvested by centrifugation in a Sharples Air Turbine Drive Centrifuge and washed three times in cold saline[2] (0.15 M NaCl) and finally suspended in cold water (0.4 g wet weight cells/ml).

↓

Step 2.

↓

E. coli 0113 cells fractionated with a Sorvall Refrigerated Cell Fractionator, Model RF-1, at 16,000 psi.

↓

Cell walls separated from the protoplasm by centrifugation in a Sorvall RC-2 Centrifuge (27,000 X g for 2 hours).

↓

Cell walls washed three times in cold water and finally suspended in 600 ml of distilled water.

↓

Step 3.

↓

600 ml of liquid phenol (Mallinckrodt Chemical Works) added to the 600 ml of cell wall suspension.

↓

Heated at 65 C for 30 minutes with stirring. A homogenous suspension resulted.

↓

continued.

Step 3--continued.

Aqueous-phenol mixture cooled to 5 C and centrifuged in an International Refrigerated Centrifuge, Model PR-6 (1700 X g for 60 minutes) to separate the aqueous and phenol phases.

Cell walls and phenol phase reextracted for endotoxin by adding 600 ml of water, heating and centrifugation as described above

Aqueous phase

Phenol phase discarded

Aqueous phases dialyzed against water at 3 C until all the phenol was removed.

Step 4.

Endotoxin was precipitated from the water by addition of sodium acetate ($NaC_2H_3O_2 \cdot 2H_2O$, Fischer Scientific Co.) to a final concentration of 0.15 M and absolute ethyl alcohol to a final concentration of 68%, while stirring at 3 C.

The solution was kept at 3 C for 24 hours to allow the endotoxin to precipitate.

Precipitated endotoxin separated from the liquid by centrifugation (1700 X g for 30 minutes).

Supernatant fluid discarded

Endotoxin pellet resuspended in 300 ml of distilled water.

Dialyzed against water at 3 C to remove traces of ethyl alcohol.

Endotoxin-water suspension lyophilyzed to yield purified endotoxin.

FIG. 1. Flow chart for the preparation of endotoxin from E. coli 0113.

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[1]M-9 medium (38) prepared by dissolving 1g NH_4Cl, 6g Na_2HPO_4, 3g KH_2PO_4, 5g NaCl, 0.1g MgS and 5% glucose in 1000 ml distilled water.

[2]All subsequent centrifugations done at 0-5 C.

TABLE 1. List of animal sera.

Class	Name of animal contributing serum: Latin name	Common name	Method of collection	Collector
Mammalia	Bos indicus	Domestic cow	Jugular puncture	Author
	Canis familiaris	Dog	x[a]	Rockland[b]
	Citellus columbianus	Columbian ground squirrel	x	RML[c]
	Cavia domestica	Guinea pig	Cardiac puncture	Author
	Citellus lateralis	Mantled ground squirrel	x	RML
	Didelphis virginianus	Oppossum	x	RML
	Erethizon dorsatum	Purcupine	x	RML
	Eutamias amoenus	Chipmunk	x	RML
	Felis domestica	Cat	x	Rockland
	Glaucomys sebrinus	Flying squirrel	x	RML
	Homo sapiens	Human		
		1	Venapuncture	Author
		2	x	RML
	Lepus americanus bairdii	Snowshoe hare	x	RML
	Marmota monax	Woodchuck	x	RML
	Mephitis mephitis	Skunk	x	RML
	Microtus pennsylvanicus	Meadow vole	x	RML
	Mus musculus	Mouse	x	RML
	Neotoma cinera	Pack rat	x	RML
	Odocoileus hemionus	Mule deer	x	RML
	Oreamous americanus	Mountain goat	x	RML
	Peromyscus maniculatus	Deer mouse	x	RML
	Procyon lotor	Raccon	x	RML
	Tamiasciurus hudsonicus	Pine squirrel	x	RML
	Sus scrofa	Domestic pig	Jugular puncture	Author
	Sylvilagus nuttali	American cottontail	x	RML
	Vulpes fulva	Red fox	x	RML

TABLE 1. Continued

Class	Name of animal contributing serum: Latin name	Common name	Method of collection	Collector
Aves	Anseriformes	Duck[d]	x	Rockland
	Anseriformes	Goose	x	Rockland
	Columbiformes	Pigeon	x	Rockland
	Galliformes	Chicken	x	Rockland
		1	Decapitation	Author
		2	x	Rockland
	Galliformes	Turkey	x	Rockland
Reptilia	Chrysemys picta	Western painted turtle	Decapitation	Author
	Thamnophis o. atratus	Garter snake	Decapitation	Author
Amphibia	Rana pipiens	Leopard frog	Decapitation	Author
Osteichthyes	Cyprinus carpio	Carp	x	CSC[e]
	Huro salmoides	Largemouth bass	Decapitation	Author
	Salmo gairdnerii	Rainbow trout	Cardiac puncture	Author
Chondrichthyes	Squalus acanthia	Spiny dog fish shark	Caudal cut	C.P.A.[f]
Crustacea	Astacus nigrescens	Crayfish	Cardiac puncture	Author
	Homarus sp.	Lobster	Cardiac puncture	J.C.[g]

TABLE 1. Continued

[a]Unknown.

[b]Rockland, Gilbertsville, Pennsylvania.

[c]Field personnel of the Rocky Mountain Laboratory, Hamilton, Montana.

[d]Genera of the sera from avian species not given by Rockland.

[e]Colorado Serum Company, Inc., Denver, Colorado.

[f]D. P. Anderson, biologist, Western Fish Disease Laboratory, Seattle, Washington.

[g]J. Cory, technician, Rocky Mountain Laboratory, Hamilton, Montana.

Heat-treatment of serum.

Several sera were examined for the capacity to alter endotoxin after they had been heated at 56 C for 30 minutes. Particulate matter was removed from the sera by centrifugation (1000 X g for 30 minutes) prior to incubation with endotoxin.

Calcium-treatment of serum.

Different amounts of calcium chloride ($CaCl_2 \cdot 2H_2O$, Fischer Scientific Co.) were added to human serum to test the effect of calcium ions on the capacity of human serum to inactivate endotoxin. Concentrations of $CaCl_2$ varying from 5 M to 0.0005 M contained in 0.02 ml were added to 0.98 ml of incubation mixture so that the final amount of calcium added was 100, 50, 10, 1, 0.1, or 0.01 micromoles.

Also, $CaCl_2$ was added to several other animal sera so that the effect of calcium ions on the capacity of these sera to alter endotoxin could be tested. In all cases, 100 micromoles (0.02 ml of a 5 M $CaCl_2$ solution) of $CaCl_2$ were added to 0.98 ml of incubation mixture. Also, 10 units of heparin (heparin-sodium, Nutritional Biochemicals Corporation) were included in each 1.0 ml of incubation mixture.

Defibrination of serum.

Some of the animal sera tested were thought to contain fibrin or fibrinogen which was causing nitrogen to be preciptated nonspecifically. To remove these clotting factors from the sera, 10 units of thrombin (Thrombin N.F., The Upjohn Company), contained in 0.02 ml of sterile saline, was added to 1.0 ml of serum. The mixtures were incubated at 4 C

for 48 hours and any fibrin which formed was removed by centrifugation (1000 X g for 30 minutes).

Collection of embryonic chicken serum.

Embryonated eggs from White Leghorn chickens were set in a humidified incubator (Buffalo Incubator Co. #6) maintained at 39 ± 1 C and were turned at least twice daily. A "batch" of eggs refers to eggs which were all set on the same day; some of each batch were removed and bled after the desired length of incubation. Several batches of eggs were used for this study and sera were collected from embryos which were incubated for varying times from 10 to 18 days. Whole blood was collected from each embryo by the following procedures. The egg was cracked open and the entire contents poured into a petri dish. The embryo was still alive at this point. The lateral vitelline vein was located and exposed to the surface by removing a section of the chorioallantoic membrane. The vein was ruptured with surgical scissors and the blood aspirated into a capillary pipet. Depending on the age, 0.2 to 0.5 ml of blood could be collected from each embryo. Blood from several embryos of the same age and belonging to the same batch of eggs was pooled, allowed to clot for 1 hour at room temperature, and refrigerated for 24 hours. The serum was separated from the clot by centifugation (1000 X g for 30 minutes) and frozen at -20 C until used for assayed.

Preparation of anti-endotoxin serum.

Four albino rabbits were injected with cells of E. coli 0113 which had been killed by boiling for 2 1/2 hours. The immunization schedule is given in Table 2. On day 49, 30 ml of whole blood was collected from

each rabbit by cardiac puncture, allowed to clot at room temperature for 1 hour, and refrigerated (4 C) for 24 hours. The serum was separated from the clot by centrifugation (1000 X g for 30 minutes), decanted into vials, and frozen (-20 C) until needed. Six days after bleeding, the rabbits were injected with 1 mg of antigen i.p. and 1 mg of antigen s.c., followed the next day by 1 mg of antigen i.v.; these rabbits were bled 7 days later.

The bleedings and injections on alternate weeks were continued for 12 months. Four antiserum pools, designated AS-1, AS-2, AS-3, and AS-4, were prepared. The sera from several bleedings were combined, heated at 56 C for 30 minutes, centrifuged (1000 X g for 30 minutes), distributed into 8 ml vials, and frozen. Serum pool AS-4 was defibrinated as described previously.

Varying amounts of endotoxin were incubated with a constant volume of homologous antiserum to determine the micrograms of nitrogen precipitated with a given amount of endotoxin. Different amounts of endotoxin, dissolved in PBS[1], were added to 0.5 ml of antiserum, 10 units of heparin (contained in 0.1 ml of distilled water) and enough PBS to give a total volume of 1.5 ml. The solutions were mixed well and incubated at 37 C for 2 hours, followed by 18 to 24 hours at 4 C. The nitrogen content of the resultant precipitates was determined as described in section 9. Curves of micrograms of nitrogen precipitated versus micrograms of endotoxin added were prepared for each antiserum pool and are given in Figure 2.

---

[1]PBS, 0.15 M NaCl; 0.0033 M phosphate; pH 7.4.

TABLE 2. Schedule used for the immunization of rabbits with killed cells of *E. coli* 0113 for the production of anti-endotoxin serum.

Day	Amount of antigen injected[a]	Route of administration
0	100 ug	Intraperitoneal (i.p.)
	100 ug	Subcutaneous (s.c.)
1	100 ug	Intravenous (i.v.)
6	250 ug	i.p.
	250 ug	s.c.
7	250 ug	i.v.
13	1 mg	i.p.
	1 mg	s.c.
14	500 ug	i.v.
20	1 mg	i.p.
	1 mg	s.c.
21	500 ug	i.v.
27	1 mg	i.p.
	1 mg	s.c.
28	1 mg	i.v.
34	1 mg	i.p.
	1 mg	s.c.
35	1 mg	i.v.
41	1 mg	i.p.
	1 mg	s.c.
42	1 mg	i.v.
49	bled	

[a]Dry weight of killed cells suspended in 1.0 ml PBS.

Incubation of endotoxin with serum.

The procedure for incubating endotoxin with sera is given in Figure 3. Proportionate changes were made in the volumes of each reactants among varying experiments but the concentrations were always the same.

The incubation procedure used for the calcium-treated sera was essentially the same as given in Figure 3, with one important change. Ten units of heparin were included in each milliliter of incubation mixture.

Quantitative precipitation assay.

Endotoxin may be precipitated by homologous antiserum, while endotoxin altered as a result of incubation with human serum will not be totally precipitated by homologous antiserum (54). Unaltered endotoxin remaining in the test incubation mixtures was precipitated with homologous antiserum in thick-walled 12 ml conical centrifuge tubes, according to the procedure described by Rudbach et al. (47) and outlined in Figure 4. Also, an antiserum blank (0.5 ml antiserum + 0.9 ml PBS + 0.1 ml heparin) was set up each time this assay was employed.

The amount of nitrogen present in the precipitate was determined by modification of the Nessler reaction (22). Duplicate tubes containing 30 ug of standard nitrogen[1] were also set up for nitrogen determination.

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[1]This is an aqueous solution of ammonium sulfate ($(NH_4)_2SO_4$, J. T. Baker Chemical Co.), containing 100 ug N/ml. 0.3 ml used for 30 ug of standard nitrogen.

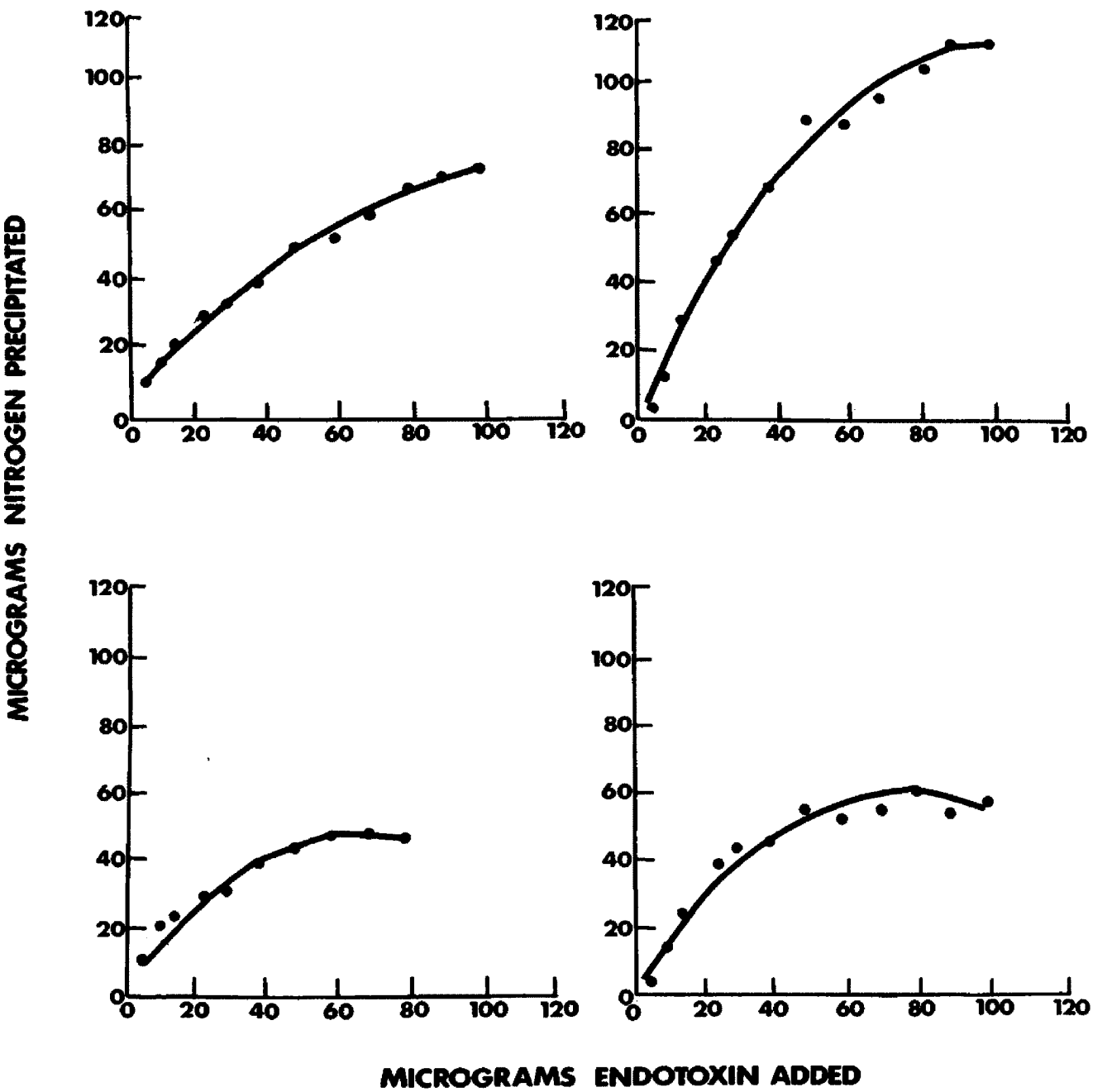

FIG. 2. Standard quantitative precipitation curves prepared with antiserum taken from rabbits immunized with heat killed cells of E. coli 0113 and endotoxin from E. coli 0113.

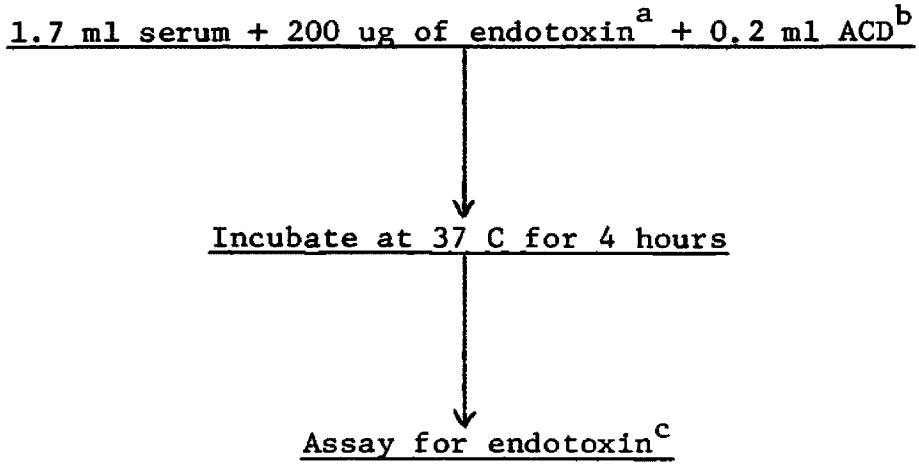

FIG. 3. Procedure used for incubating endotoxin with serum.

[a]Endotoxin was suspended in PBS to a final concentration of 2 mg/ml. 0.1 ml was used to give the incubation mixture a final concentration of 100 ug endotoxin/ml incubation mixture.

[b]ACD, 22.0 g tri-sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$, Allied Chemical), 8.0 g citric acid ($HOC(COOH)(CH_2COOH)_2$, J. T. Baker Chemical Co.), 22.2 g dextrose ($C_6H_{12}O_6$, Mallinckrodt Chemical Works).

[c]The incubation mixtures were assayed for endotoxin immediately following incubation or frozen (-20 C) until assays could be performed.

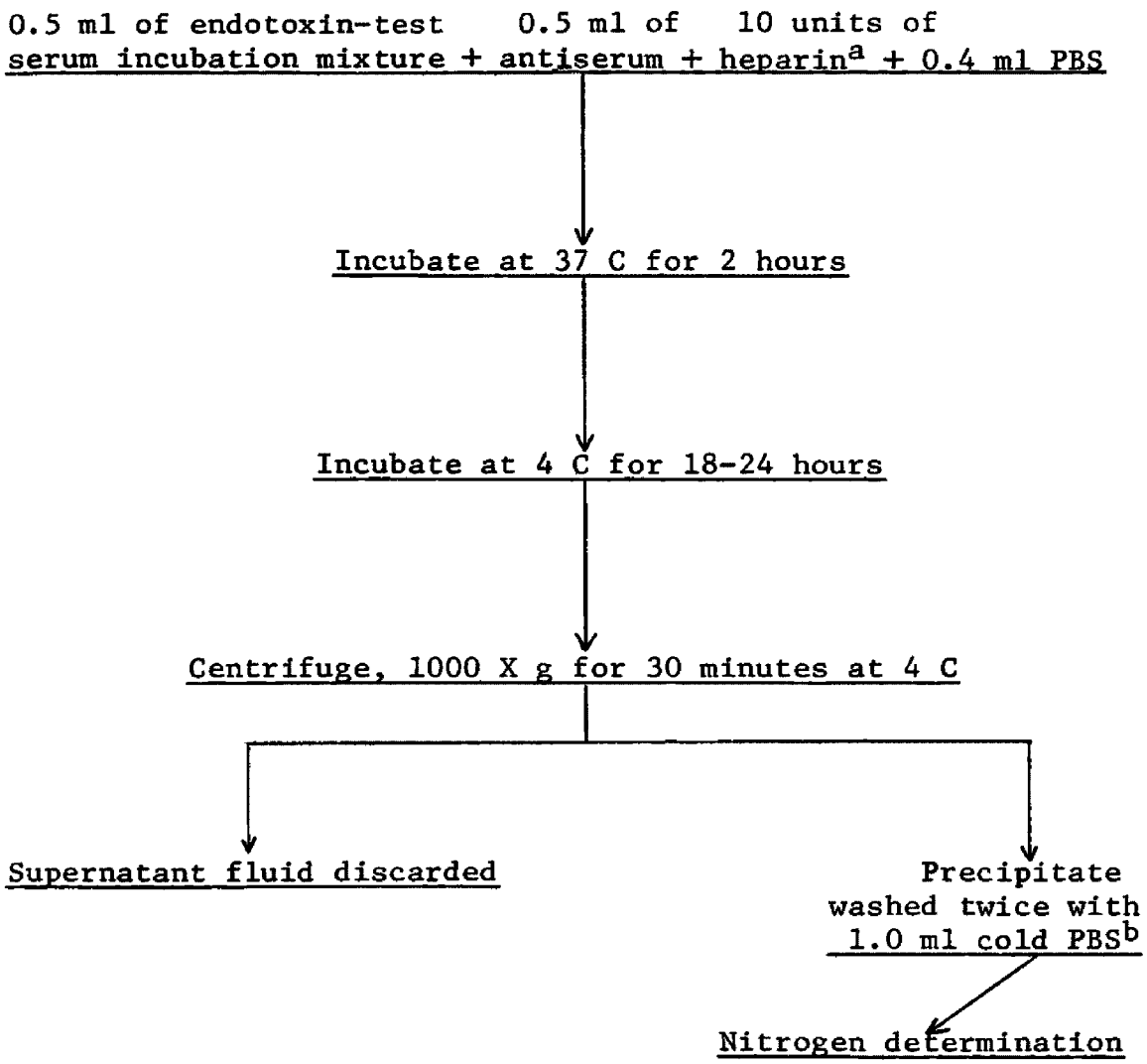

FIG. 4. Procedure used for the quantitative precipitation of endotoxin by homologous antiserum.

[a]Contained in 0.1 ml of distilled water.

[b]The precipitates were suspended in 1 drop of cold PBS by vigorous mixing, followed by addition of 1.0 ml of cold PBS, and then separated from the supernatant fluid by centrifugation (1000 X g for 15 minutes). The precipitates were kept in an ice bath when not under refrigeration.

To each tube 0.2 ml of digestion mixture[2] was added and the samples were digested by placing the tubes in a cold tube furnace and allowing the temperature to reach 280 C. The tubes were removed and allowed to cool. One drop of 30% hydrogen peroxide was added to each, following which they were heated at 280 C for 30 minutes. If the digest appeared colored at this point, 1 more drop of 30% H_2O_2 was added and heating was continued. The tubes were allowed to cool and 2.0 ml of distilled water was added. The tubes were mixed and 2.0 ml of Nessler's reagent[3] was added. Following another mixing, 3.0 ml of 2N NaOH was added and the tubes were again mixed and allowed to stand for 15 minutes at room temperature. The absorbancy was determined for each sample in a Klett-Summerson Colorimeter, Model 800-3 with a #50 green filter. The instrument had been zeroed with a reagent blank (0.2 ml digestion mixture + 2.0 ml distilled water + 2.0 ml Nessler's reagent + 3.0 ml 2N NaOH). In most cases duplicate Klett values were obtained for each test serum from which averages were calculated. The quantity of nitrogen present in the precipitates was calculated, by comparison to the standard preparation of 30 ug of nitrogen, according to the following forumla:

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[2]Digestion mixture: 0.181 g sodium selenite ($Na_2SeO_3 \cdot 5H_2O$, J. T. Baker Chemical Co.), 0.262 g copper sulfate pentahydrate[3] ($CuSO_4 \cdot 5H_2O$, J. T. Baker Chemical Co.), and 56.0 ml concentrated sulfuric acid (H_2SO_4, J. T. Baker Chemical Co.). Dissolve the solids separately in water and then mix. A green gelatinous suspension forms which clears upon the addition of the acid. Then add water to 200 ml.

[3]Nessler's reagent: Dissolve 5 g of potassium iodide (KI, J. T. Baker Chemical Co.) and 5 g of mercuric iodide (HgI, J. T. Baker Chemical Co.) in 25 ml of water. Add this solution to 750 ml of warm gum ghatti (Fischer Scientific Company) solution prepared by boiling 1.75 g of the powdered gum with 750 ml of water for 4 hours. The resultant mixture is allowed to stand for 3 days and then is filtered through a thick layer of glass wool on a Buchner funnel. Dilute the filtrate to 1 liter with water.

$$\text{ug nitrogen in the precipitate} = \frac{\text{Corrected Klett reading of the test precipitate}}{\text{Klett reading of the standard 30 ug of nitrogen}} \times 30$$

The nitrogen content of the antiserum blank was subtracted from the experimental values to correct for nonspecific nitrogen contributed by the antiserum and the reagents. The ug of precipitated nitrogen was converted to ug of endotoxin by interpolation from the standard curves previously presented (Fig. 2).

The same concentration of endotoxin incubated with test serum was incubated with PBS. The ug of unaltered endotoxin in the endotoxin-PBS incubation mixture was determined by precipitation with homologous antiserum as described above. This value was taken as 100% recovery of the endotoxin. The percent inactivation of endotoxin by the test serum was determined with the following formula:

$$\% \text{ Inactivation} = 100\left(1 - \frac{\text{ug of endotoxin recovered from test serum}}{\text{ug of endotoxin recovered from PBS}}\right)$$

Immunodiffusion assay.

The Ouchterlony method (33) of immunodiffusion was used to analyze the precipitation patterns of varying incubation mixtures with anti-endotoxin serum. Molten agar[1] was allowed to solidify in a petri dish. Patterns were cut in the agar with the aid of a plexiglass template and a circular punch. Endotoxin-test serum incubation mixtures were placed in the antigen wells and antiendotoxin serum was added to the antibody

[1] 0.5% agar was made by adding 4.37 g sodium chloride (NaCl, J. T. Baker Chemical Co.), 2.5 g Ionagar (No. 2, Consolidated Laboratories, Inc.), and 50 mg merthiolate (Powder No. 20, Eli Lilly Co.) to 500 ml of distilled water. The mixture was heated at 121 C for 15 minutes, distributed 12 ml/tube, and refrigerated (4 C).

wells, The plates were kept in a humid chamber for 48 hours at room temperature and the precipitation patterns recorded photographically while the plates were illuminated with oblique light. The presence of the band of precipitate representative of the slowly migrating antigen of biologically active endotoxin (44) was recorded as a plus test, whereas a negative test indicated the complete lack of this band of precipitate.

Mouse lethality test.

Increase in LD_{50} of endotoxin following incubation in serum was determined with Rocky Mountain Laboratory white mice, 3 to 6 weeks old. Endotoxin and test serum were combined and incubated as described previously (Fig. 3), with the volume of each reactant increased by a factor of 4. Serial two-fold dilutions, in saline, were made from the original incubation mixture to a 1/128 dilution. The lethal effect of endotoxin was potentiated with lead acetate, a procedure described by DeClercq and Merigan (10). Lead acetate ($Pb(CH_3CO_2)_2 \cdot 3H_2O$, J. T. Baker Chemical Co.) was dissolved in deionized water (5 mg/ml) and 0.2 ml (1 mg) was injected i.v. into each mouse. Approximately 15 minutes following the lead acetate, each mouse was injected i.p. with 0.5 ml of the endotoxin dilution. Five mice were used for each dilution of endotoxin. The following controls were employed each time this assay was used: 1) five mice receiving only 0.2 ml each of lead acetate i.v.; 2) five mice receiving 0.2 ml lead acetate i.v. and 0.5 ml saline i.p.; and 3) when a sufficient volume of normal serum was available, five mice received 0.2 ml lead acetate i.v. and 0.5 ml undilute normal serum i.p.

In most cases there were no deaths of the control mice. Rarely, one of the lead acetate-saline control mice would die. Only one animal serum tested, undilute turtle serum heated at 56 C for 30 minutes, was lethal for the mice.

Deaths were recorded daily for three days, and most deaths occurred within 48 hours of injection. The LD_{50} was determined for each test incubation mixture according to the method of Reed and Meunch (2). Also, the LD_{50} for endotoxin incubated in PBS was determined each time this assay was used. This latter value was taken as 100% recovery of the endotoxin. The percent inactivation of endotoxin as a result of incubation with the test serum was calculated according to the following formula:

$$\% \text{ Inactivation} = 100\left(1 - \frac{LD_{50} \text{ of endotoxin incubated in PBS}}{LD_{50} \text{ of endotoxin incubated in test serum}}\right)$$

Chicken embryo lethality assay ($CELD_{50}$).

The chicken embryo lethality assay was performed[1] as described by Milner and Finkelstein (29). Fertilized eggs were incubated for 11 days at 38 $\pm$ 1 C. Five-fold dilutions were made from an initial 1/100 dilution of the incubation mixtures in saline containing 0.2% formalin and 0.1 ml of test dilution was injected i.v. into each embryo. Ten embroys were used for each dilution. Deaths were recorded 24 hours after injection and the LD_{50} determined for each test mixture (29). In some cases fertile eggs of different ages were injected to test the change

[1]This assay was performed by Dr. K. C. Milner of the Rocky Mountain Laboratory, Hamilton, Montana, on preparations supplied to him by the author.

in susceptibility of the maturing chicken embryo to the lethal effect of endotoxin.

Limulus lysate assay.

A modification of the Limulus lysate assay described by Reinhold and Fine (35) was used to test the inactivation of endotoxin by embryonic chick sera. The lysate of amebocytes from the horseshoe crab (Limulus polyphemus) was generously supplied by Dr. J. Fine and kept frozen (-20 C) until used. Pyrogen free test tubes were prepared by heating clean, capped tubes at 270 C for 2 hours. Serial two-fold dilutions, in deionized water, were made from the original incubation mixtures (previously described, Fig. 3) to a 1/1024 dilution and 0.1 ml of each dilution was placed in a tube followed by 0.1 ml of Limulus lysate. The tubes were then incubated in a 37 C water bath for 45 minutes. The presence of increased viscosity or solid gel formation upon inversion of the tube was recorded as a positive test for the presence of endotoxin. Tests which showed no increased viscosity or gelation were recorded as negative. The following controls were used each time this assay was employed: 1) 0.1 ml of Limulus lysate + 0.1 ml of 100 ug endotoxin/ml PBS; 2) 0.1 ml of Limulus lysate + 0.1 ml of deionized water; and 3) 0.2 ml Limulus lysate. Control #1 showed gelation in all cases. If control #2 revealed a positive test, and this occurred occasionally, the results of the entire assay were discarded. Control #3 was negative in all cases.

The concentration of endotoxin following incubation with a test serum was calculated for each serum using the formula given by Reinhold and Fine (35):

$$(ETOX)_o = (ETOX)_{ep}/Dilution,$$

where $(ETOX)_o$ = concentration of endotoxin in original sample; $(ETOX)_{ep}$ = lowest concentration of endotoxin giving a positive gelation as determined by standards; and Dilution = highest dilution of test sample giving a positive test for endotoxin. Serial two-fold dilutions of endotoxin incubated in PBS were used to determine the lowest concentration of endotoxin which gave a positive test for endotoxin. The percent inactivation of endotoxin by incubation with a test serum was then calculated as follows:

$$\% \text{ Inactivation} = 100 \left(1 - \frac{\text{Concentration of endotoxin added to test serum prior to incubation}}{\text{Concentration of endotoxin in test serum following incubation}}\right)$$

CHAPTER III

RESULTS

The capacity of normal animal sera to inactivate endotoxin.

The initial studies were designed to test the capacity of sera obtained from several classes of animals to inactivate endotoxin. It was first necessary to ascertain the variability that could be expected in the assays. Due to limited volumes of sera, only results obtained by the quantitative precipitation assay could be used for statistical analysis. These results are given in Table 3. Taking into account both variation among replicate assays of the same serum sample and variation in the percent inactivation of endotoxin by several serum samples of the same species, values for the percent inactivation of endotoxin falling in the range of 50 to 100% were not considered different from each other. That is to say, serum samples which gave a percent inactivation in this range have a quantitatively similar capacity to inactivate endotoxin.

With this established, sera from several mammalian species were tested for the capacity to inactivate endotoxin. The results are presented in Table 4. With one exception, all mammalian sera tested inactivated endotoxin to approximately the same extent as determined by the quantitative precipitation assay. Unfortunately, only enough serum from the mule deer was available for one assay, and the significance of this low percentage of inactivation cannot be evaluated. It should be noted that in most cases there was good correlation between the percent

TABLE 3. Comparing the capacity of sera from members of the same species to inactivate endotoxin.

Type of serum[a] incubated with endotoxin		% Inactivation of endotoxin[b]	Mean ± 2 SD[c]	
Animal	Serum sample			
Human	1	82 (17)[d]	82 ± 22.7	
	2	89 (1)		
	3	82 (1)		
	4	76 (1)		72.6 ± 22.8
	5	67 (1)		
	6	60 (1)		
	7	58 (3)		
Cow	1	67 (1)		
	2	61 (1)		ND[e]
	3	53 (1)		
Pig	1	83 (1)		
	2	81 (1)		
	3	75 (1)		
	4	71 (1)		73.5 ± 15.2
	5	67 (1)		
	6	64 (1)		
Turtle	1	74 (1)		ND
	2	72 (1)		

[a]Each serum sample contained 15 ml ACD/100 ml of serum.

[b]As determined by the quantitative precipitation assay.

[c]SD = standard deviation.

[d]Number of results used to average the % inactivation value.

[e]ND = not done.

TABLE 4. Capacity of sera from mammalian species to inactivate endotoxin.

Type of serum[a] incubated with endotoxin		% Inactivation of endotoxin[b]			Immuno-diffusion assay
Order	Common name	Quantitative precipitation		Mouse lethality	
Primate	Human	78	(7)[c]	82	-[e]
Carnivora	Raccoon	99	(1)	ND[d]	-
	Red fox	91	(1)	68	-
	Skunk	80	(1)	ND	-
	Dog	61	(2)	ND	-
	Cat	50	(2)	92	-
Artilodactyla	Mountain goat	73	(1)	ND	-
	Domestic pig	73	(6)	53	-
	Domestic cow	62	(4)	62	-
	Mule deer	32	(1)	31	-
Lagomorpha	Snowshoe hare	80	(1)	ND	-
	American cottontail	75	(1)	88	-
Rodentia	Chipmunk	100	(1)	ND	-
	Flying squirrel	98	(2)	ND	-
	Guinea pig	96	(3)	ND	-
	Mantled ground squirrel	92	(1)	ND	-
	Woodchuck	92	(1)	ND	-
	Porcupine	92	(2)	90	-
	Pine squirrel	89	(1)	ND	-
	Mouse	88	(1)	ND	-
	Pack rat	88	(1)	33	-
	Columbian ground squirrel	83	(1)	66	-
	Meadow vole	83	(1)	81	-
	Deer mouse	61	(1)	84	-
Marsupialia	Oppossum	87	(1)	ND	-

[a]Each serum sample contained 15 ml ACD/100 ml serum.

[b]Determined by comparison to endotoxin incubated with phosphate-buffered saline (PBS).

[c]Number of results used to average the % inactivation value.

[d]Not done.

[e]Negative symbol (-) indicates the complete lack of the slowly migrating antigen associated with the biologically active endotoxin. A positive symbol (+) indicates the presence of the slowly migrating antigen.

inactivation of endotoxin as determined by the quantitative precipitation assay and mouse lethality assay. Furthermore, all sera appeared to inactivate endotoxin when inactivation was tested by the immunodiffusion assay.

Lesser degrees of inactivation of endotoxin were found when sera from avian species were tested (Table 5). As determined by the t test for nonpaired experiments (3), the mean percentage of inactivation of endotoxin by avian sera was significantly (0.001) less than that given by human serum. However, examination of the sources of avian sera revealed that with one exception all of the sera came from a single commercial source. Also, the chicken serum from the commercial supplier (Rockland) had a lesser capacity to inactivate endotoxin than chicken sera collected by the author. Despite the results of the t test, the conclusion could not be made that avian serum had a different capacity to inactivate endotoxin than did human serum.

Normal sera from poikilothermic species of vertebrates were also shown to possess the capacity to alter endotoxin (Table 6), with one exception. Normal serum from the dogfish shark did not inactivate endotoxin as shown by the mouse lethality test and immunodiffusion assay, and had minimal inactivation of endotoxin as determined by the quantitative precipitation assay. These sera from poikilothermic vertebrates, excluding sera from the shark, appeared to have a capacity for altering endotoxin quantitatively similar to human serum.

The finding that normal shark serum did not inactivate endotoxin while sera from species of ostheichthyes could inactivate endotoxin suggested that the evolutionary origin of the endotoxin altering

TABLE 5. Capacity of sera from avian species to inactivate endotoxin.

Type of serum[a] incubated with endotoxin		% Inactivation of endotoxin[b]			Mean ± 2SD[c]
Order	Common name	Quantitative precipitation	Mouse lethality	Immuno-diffusion assay	
Anseriformes	Goose	50 (1)[d]	53	-[e]	49.2 ± 28.6
	Duck	45 (1)	ND[f]	-	
Columbiformes	Pigeon	75 (1)	13	-	
Galliformes	Chicken	51[g] (5)	70	-	
	Turkey	42 (2)	77	-	

[a]Each serum sample contained 15 ml ACD/100 ml of serum.

[b]Determined by comparison to endotoxin incubated in PBS.

[c]SD = standard deviation.

[d]Number of results used to average the % inactivation value.

[e]Negative symbol indicates the complete lack of the slowly migrating antigen associated with biologically active endotoxin; positive symbol indicates the presence of the slowly migrating antigen.

[f]ND = not done.

[g]Commercial serum averaged 48% inactivation, chicken serum collected by the author averaged 63% inactivation.

TABLE 6. Capacity of sera from poikilothermic species to inactivate endotoxin.

Type of serum[a] incubated with endotoxin		% Inactivation of endotoxin[b]			Immuno-diffusion assay
Order	Common name	Quantitative precipitation		Mouse lethality	
	Vertebrates				
Reptilia	Western painted turtle	73	(3)[c]	63	-[d]
	Garter snake	80	(1)	ND[e]	-
Amphibia	Leopard frog	77	(1)	ND	-
Osteichthyes	Carp	86	(2)	ND	-
	Large-mouth bass	84	(1)	ND	-
	Rainbow trout	ND		63	-
Chondrichthyes	Dogfish shark	28	(2)	0	+
	Invertebrates				
Crustacea	Lobster	0	(2)	0	+
	Crayfish	0	(1)	ND	+

[a]Each serum sample contained 15 ml ACD/100 ml serum.
[b]Determined by comparison to endotoxin incubated in phosphate-buffered saline.
[c]Number of results used to average the % inactivation value.
[d]Negative symbol (-) indicates the complete lack of the slow migrating antigen associated with the biologically active endotoxin. A positive symbol (+) indicates the presence of the slow migrating antigen.
[e]Not done.

mechanism was to be found somewhere among the more primitive vertebrates. Accordingly, sera from the animals belonging to the most primitive class of vertebrate, Cyclostomata, should have been tested as these animals gave rise to both the Chondrichthyes and Osteichthyes (cf. Fig. 5.). Unfortunately, repeated attempts to locate a source of serum from any of the cyclostomes failed. However, the hemolymphs of two species of invertebrates were available to test for the capacity to inactivate endotoxin. As shown in Table 6, hemolymphs from these species were incapable of inactivating endotoxin.

The heat stability of the endotoxin altering mechanism.

The stability of the endotoxin altering mechanism was tested by heating several types of sera at 56 C for 30 minutes prior to incubation with endotoxin. The results are given in Table 7. Heat treatment did not change the capacity of sera from homoiothermic animals to inactivate endotoxin. The capacity to alter endotoxin by sera from two poikilothermic animals, the turtle and carp, appeared to be reduced by heating the sera. However, serum from another poikilothermic animal, the dogfish shark, appeared to increase in the capacity to inactivate endotoxin as a result of heat treatment. Therefore, lobster hemolymph, normally incapable of inactivating endotoxin, was heated and then tested for the capacity to inactivate endotoxin. Unlike the shark serum, no endotoxin altering activity could be demonstrated by heated lobster hemolymph. The capacity of heated shark and lobster serum to inactivate endotoxin was also tested by the mouse lethality assay and the results correlated well with the results given by the quantitative precipitation assay (Table 7).

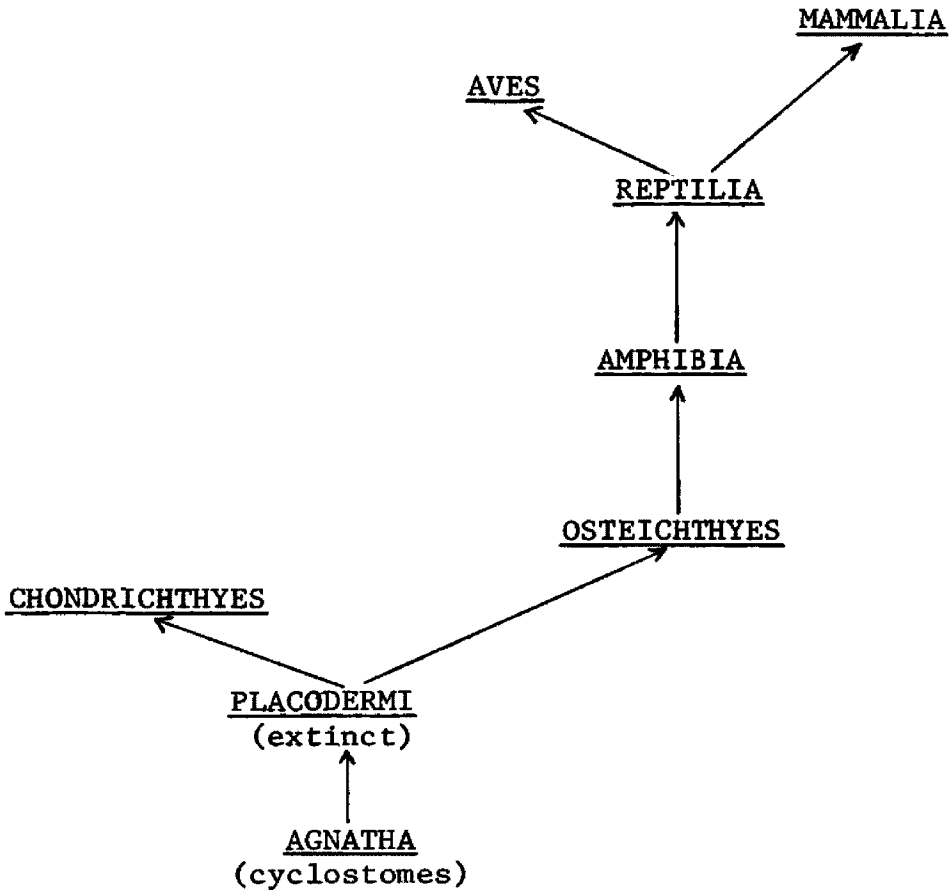

FIG. 5. Schematic drawing of the evolution of the classes of vertebrates. (Modified from Romer, 40.)

TABLE 7. Effect of heat (56 C for 30 minutes) on the capacity of sera from several animals to alter endotoxin.

Type of serum[a] incubated with endotoxin		% Inactivation of endotoxin[b]		Immunodiffusion assay	
Class	Animal species	Unheated	Heated	Unheated	Heated
Mammalia	Raccoon	99	84	-[c]	-
	Flying squirrel	98	88	-	-
	Guniea pig	96	89	-	-
	Porcupine	92	87	-	-
	Red fox	91	82	-	-
	Mouse	89	66	-	-
	Oppossum	88	82	-	-
	Rabbit	89	64	-	-
	Snowshoe hare	80	77	-	-
	Mountain goat	73	65	-	-
	Human	73 (82)[d]	82 (91)	-	-
	Pig	73	82	-	-
	Cow	62	82	-	-
	Deer	32	49	-	-
Aves	Pigeon	75	58	-	-
	Goose	50	45	-	-
	Duck	45	58	-	-
	Turkey	42	58	-	-
Reptilia	Turtle	73	0	-	+
Osteichthyes	Carp	86	39	-	-
Chondrichthyes	Dogfish shark	28 (0)	58 (51)	+	-
Crustacea	Lobster	0 (0)	5 (0)	+	+

[a]Each serum sample contained 15 ml ACD/100 ml serum.

[b]As determined by the quantitative precipitation assay.

[c]Negative symbol (-) indicates the complete lack of the slowly migrating antigen associated with biologically active endotoxin; positive symbol (+) indicates the presence of the slowly migrating antigen.

[d]Number in parentheses gives percent inactivation of endotoxin as determined by the mouse lethality assay.

The effect of temperature of incubation on the capacity of animal sera to inactivate endotoxin

The variation in the heat stability of the endotoxin altering system between sera from homoiothermic and poikilothermic animals suggested the following experiment. Sera from three homoiothermic species and three poikilothermic species were incubated with endotoxin at 3 C, 26 C, and 37 C. It was found that higher temperatures, in general, resulted in greater inactivation of endotoxin (Figure 6). The exception was lobster hemolymph which did not inactivate endotoxin at any of the temperatures of incubation tested.

Effect of excess calcium on the capacity of animal sera to inactivate endotoxin

For the most part, the results presented above suggested analogous mechanisms of endotoxin alteration in the mammalian and avian sera. Therefore, in an attempt to correlate further the mechanisms of endotoxin alteration in sera from several animals, another characteristic of the endotoxin altering system was examined. Previous work (26, 41, 51, 52) has shown that sufficient concentrations of divalent cations, most notably calcium, inhibited the capacity of human serum to inactivate endotoxin. These results were confirmed and the amount of calcium necessary for inhibition of the endotoxin altering capacity of human plasma was determined (Figure 7). It was found that 100 micromoles of calcium was the minimal amount which completely inhibited the capacity of human plasma to inactivate endotoxin. Subsequently, this amount of calcium was added to a variety of animal sera which were then tested for endotoxin altering activity. The results are given in

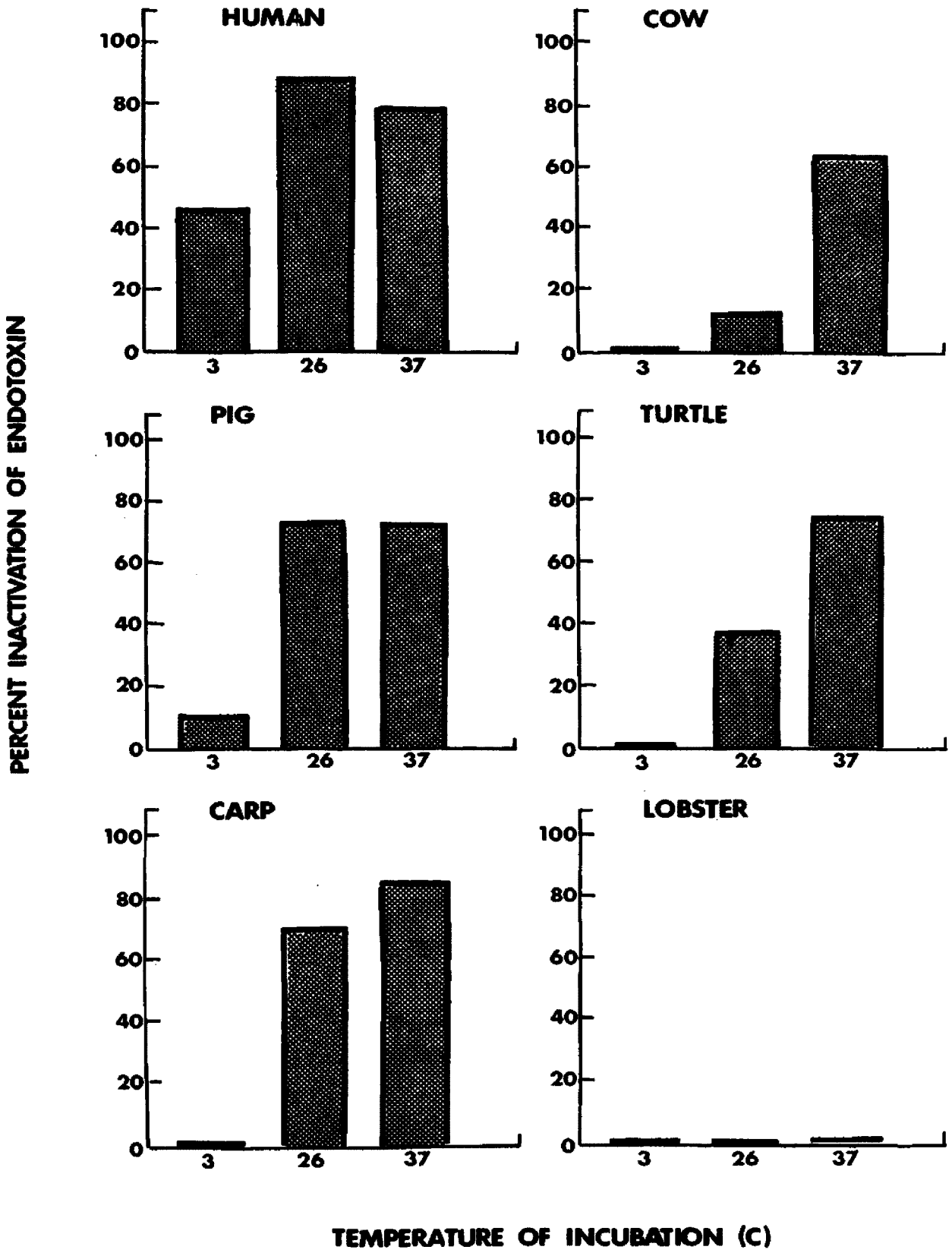

FIG. 6. The effect of the temperature of incubation on the ıimal sera to inactivate endotoxin. Percent inactiva- :d by the quantitative precipitation assay. Except for ıcubation temperatures, the incubation mixtures were : standard manner.

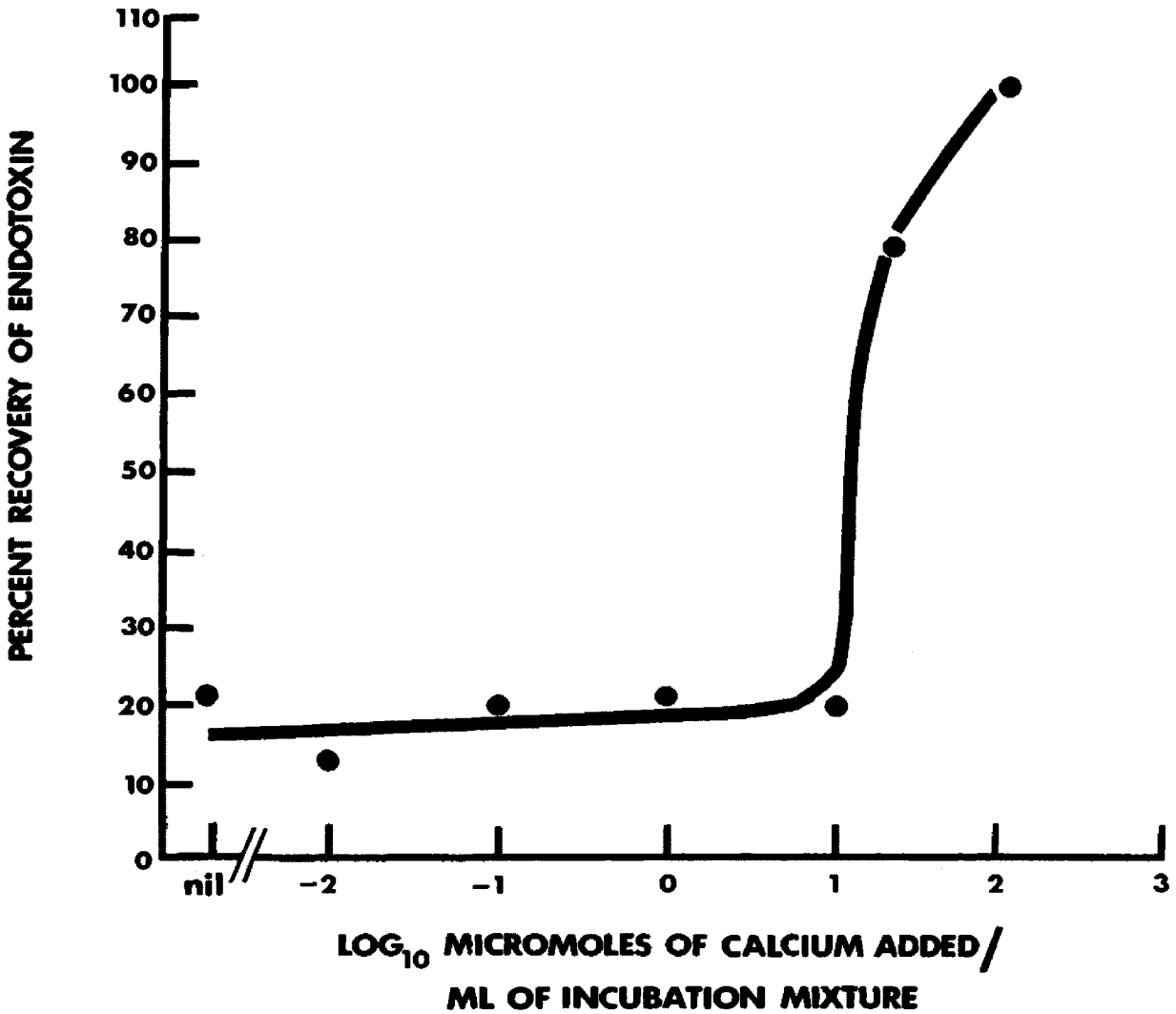

FIG. 7. The effect of calcium ions on the capacity of normal human plasma to inactivate endotoxin, as determined by the quantitative precipitation assay. Calcium chloride, dissolved in deionized water, was used as the source of calcium ions. The human serum originally contained 15 ml ACD/100 ml of serum as an anticoagulant.

Table 8. Calcium reduced the endotoxin altering capacity of all sera tested. This inhibition was demonstrated by both the quantitative precipitation and immunodiffusion assays, thus substantiating the concept that similar mechanisms were operating during the inactivation of endotoxin by the various sera.

The capacity of sera to inactivate endotoxin as determined by immunodiffusion assay

Previously, the results of the immunodiffusion assay have been described as either positive or negative. It should be pointed out that regardless of the source of the sera used for inactivation of endotoxin the immunodiffusion patterns of the altered endotoxin (negative symbol) were similar. Altered endotoxin consistantly precipitated in a band located approximately midway between the antigen and antibody wells and this band of precipitate formed a line of identity with the band of precipitate given by endotoxin altered by human serum. Examples of this pattern are shown by the bands of precipitate corresponding to wells 3 and 4 of Figure 8. Also, the immunodiffusion patterns of endotoxin which was not inactivated (positive symbol) by various incubation mixtures were also similar, and are exemplified by the bands of precipitate corresponding to wells 1, 2, and 6 of Figure 8. An analogous mechanism of inactivation of endotoxin by a variety of sera was again indicated by the results of this assay.

TABLE 8. Effect of calcium on the ability of sera from several animal classes to alter endotoxin.

Type of serum incubated with endotoxin		% Inactivation of endotoxin[a]		Immunodiffusion assay	
Class	Animal species	Normal[b]	Calcium[c]	Normal	Calcium
Mammalia	Chipmunk	100	21	-[e]	+
	Raccoon	98	0	-	+
	Flying squirrel	98	0	-	+
	Ground squirrel	92	24	-	+
	Woodchuck	92	14	-	+
	Porcupine	92	15	-	+
	Red fox	91	28	-	+
	Mouse	88	47	-	+
	Oppossum	88	10	-	+
	Pack rat	88	22	-	+
	Meadow vole	83	17	-	+
	Snowshoe hare	80	16	-	+
	Human	73	0	-	+
	Mountain goat	73	0	-	+
	Cow	62	2	-	+
	Deer mouse	61	0	-	+
	Deer	32	0	-	+
Aves	Pigeon	75	0	-	+
	Chicken	51	17	-	+
	Duck	45	17	-	+
	Turkey	42	11	-	+
Reptilia	Turtle	73	0	-	+
Chondrichthyes	Dogfish shark	58[d]	22[d]	-	+

[a]As determined by the quantitative precipitation assay.

[b]Each serum sample contained 15 ml ACD/100 ml serum.

[c]100 umoles of calcium added/ml of incubation mixture.

[d]The values for the shark serum were obtained using serum which was heated at 56 C for 30 minutes prior to incubation with endotoxin.

[e]Negative symbol (-) indicates the complete lack of the slowly migrating antigen associated with biologically active endotoxin; positive (+) symbol indicates the presence of the slowly migrating antigen.

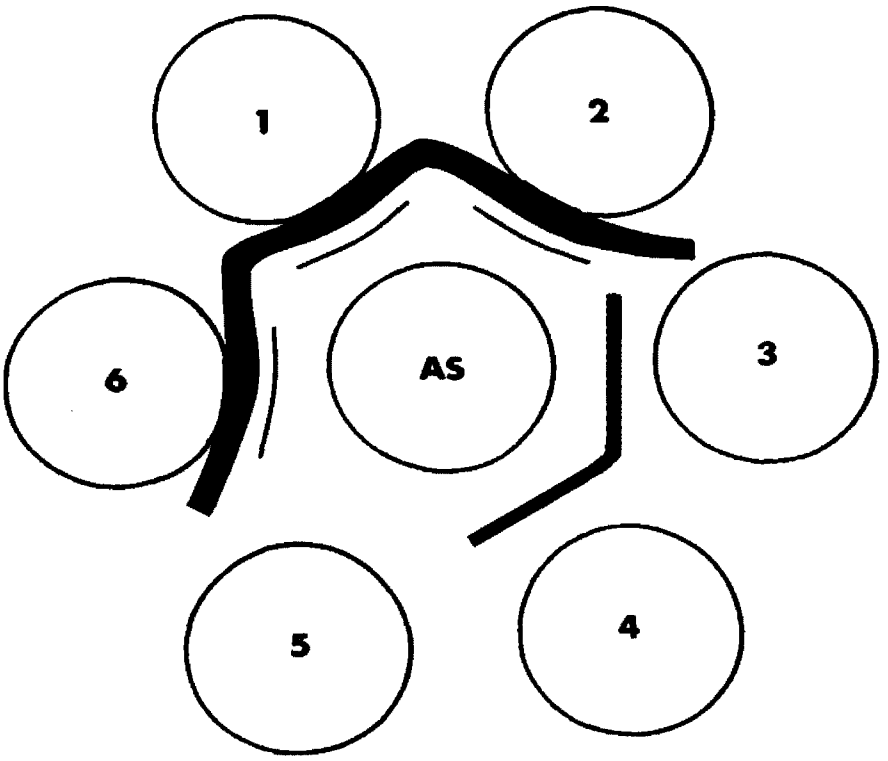

FIG. 8. Drawing of an immunodiffusion plate containing antiserum to E. coli 0113 endotoxin in the center well(AS); endotoxin incubated with PBS in wells 1 and 6; endotoxin incubated with normal shark serum in well 2; endotoxin incubated with heated (56 C for 30 minutes) shark serum in well 3; endotoxin incubated with normal human serum in well 4; and PBS in well 5.

Examination of the susceptibility of chicken embryos to endotoxin

The change in susceptibility of the chicken embryo to the lethal effects of intravenous injection of endotoxin has been reported (15, 16). Experiments were designed in an attempt to correlate these *in vivo* results with the *in vitro* inactivation of endotoxin by incubation with the embryonic serum. First, to determine the susceptibility of embryos to intravenous injection of endotoxin on a day-to-day basis, 11- to 14-day old embryos were injected with varying amounts of endotoxin and the LD_{50} determined for each age. A marked change in susceptibility occurred between the 12th and 14th day of incubation (Figure 9).

Examination of the capacity of sera from chicken embryos to inactive endotoxin

Sera were then collected from 11- to 18-day old embryos and tested for capacity to inactivate endotoxin as measured by the quantitative precipitation assay. The results are given in Figure 10. There appeared to be an increase in the capacity of sera to inactivate endotoxin as the embryo matured from day 11 to day 13.

A somewhat more dramatic increase in the capacity of sera from embryos of varying ages to inactivate endotoxin was observed when the incubation mixtures were assayed with *Limulus* lysate (Figure 11). Change in the capacity of sera to alter endotoxin appeared to occur most markedly between day 10 and day 12 of incubation.

Further tests of the capacity of sera from chicken embryos of varying ages to inactivate endotoxin, as assayed by chick embryo lethality and mouse lethality, indicated that serum from 10-day old embryos possessed the capacity in inactivate endotoxin (Table 9), and that, although sera from older embryos seemed to be slightly more effective in this regard, the differences were marginal.

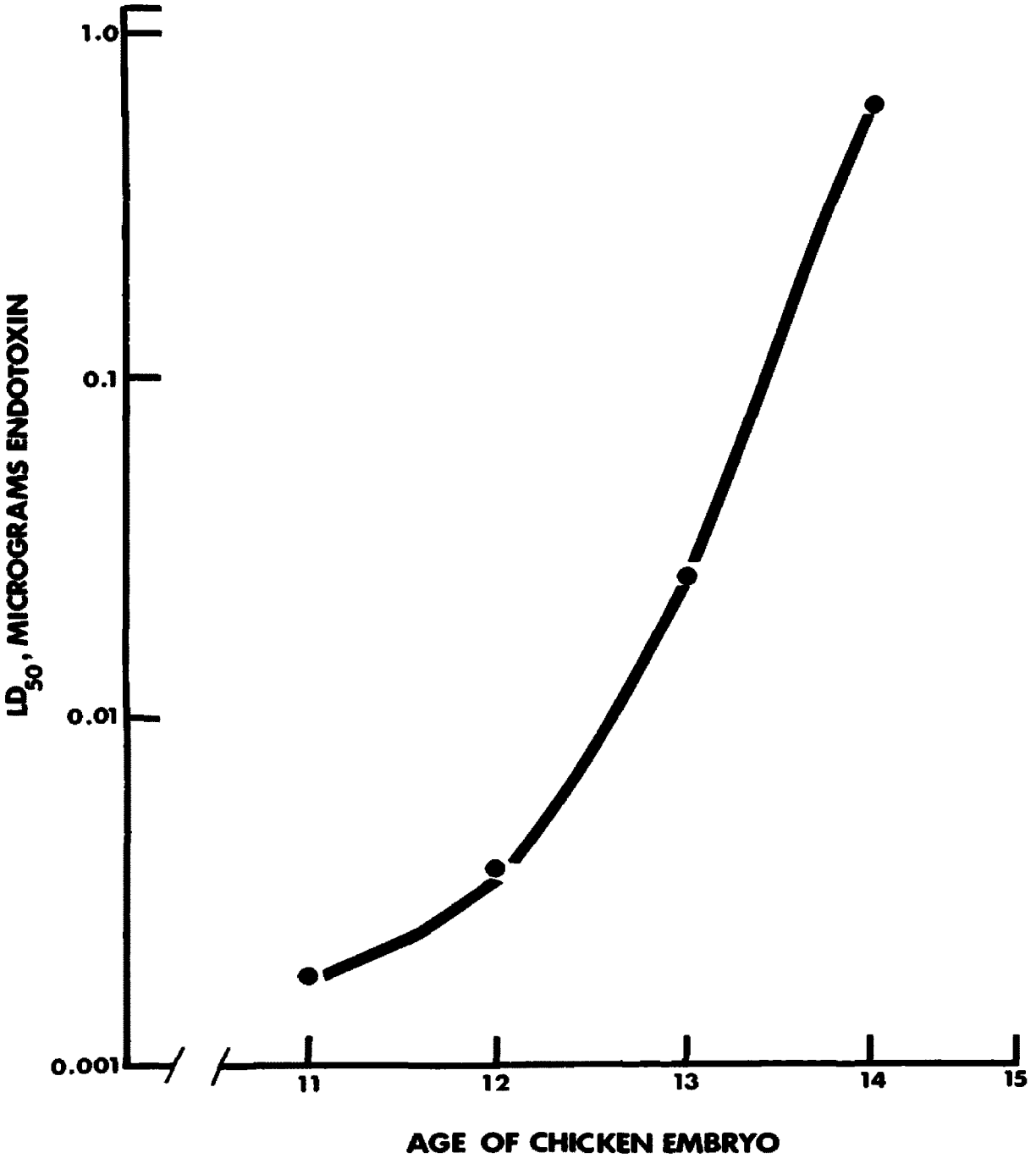

FIG. 9. The susceptibility of chicken embryos of different ages to the lethal effects of intravenous injection of endotoxin. LD_{50} for embryos of each age determined by the $CELD_{50}$ assay, performed in the laboratory of Dr. Kelsey Milner.

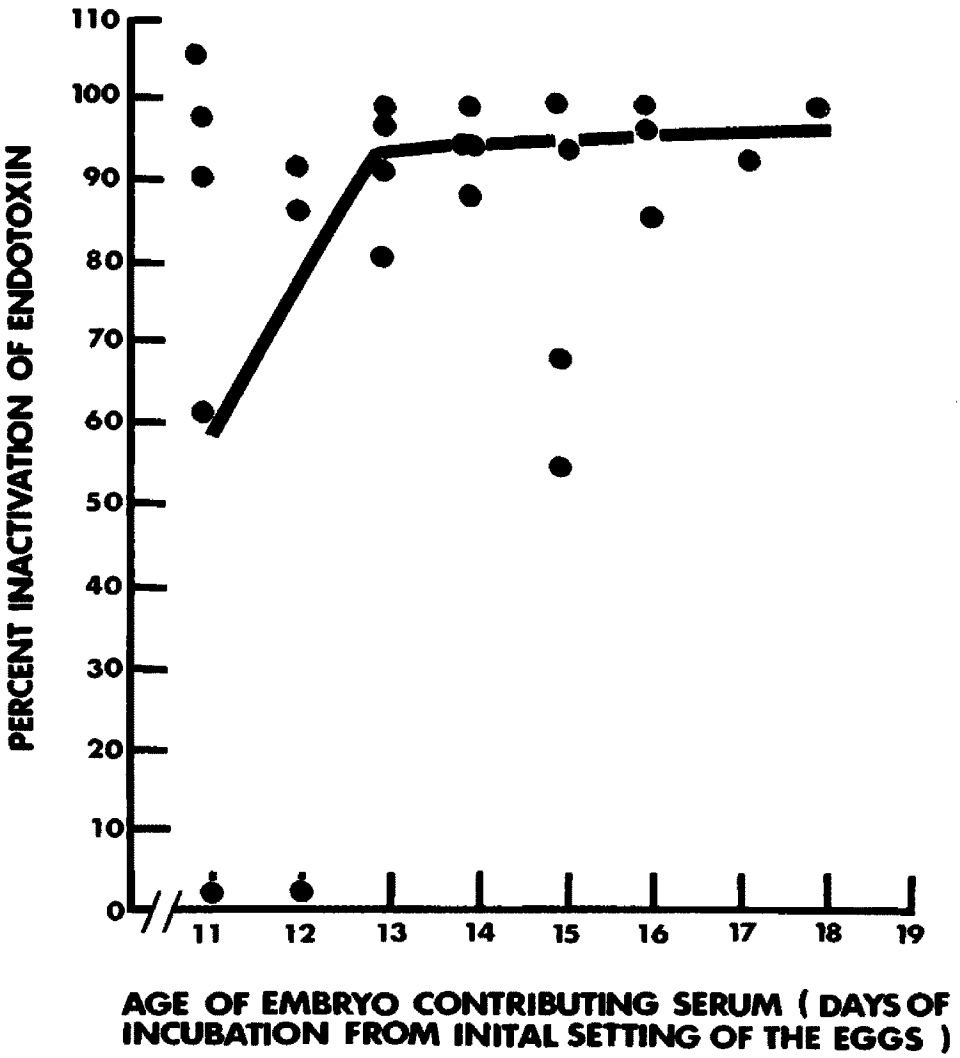

FIG. 10. The capacity of sera from chicken embryos of different ages to inactivate endotoxin. Percent inactivation determined by the quantitative precipitation assay. The experiment was repeated with several batches of eggs. Each point gives the value obtained with sera pooled from embryos of the same age and belonging to one batch of eggs.

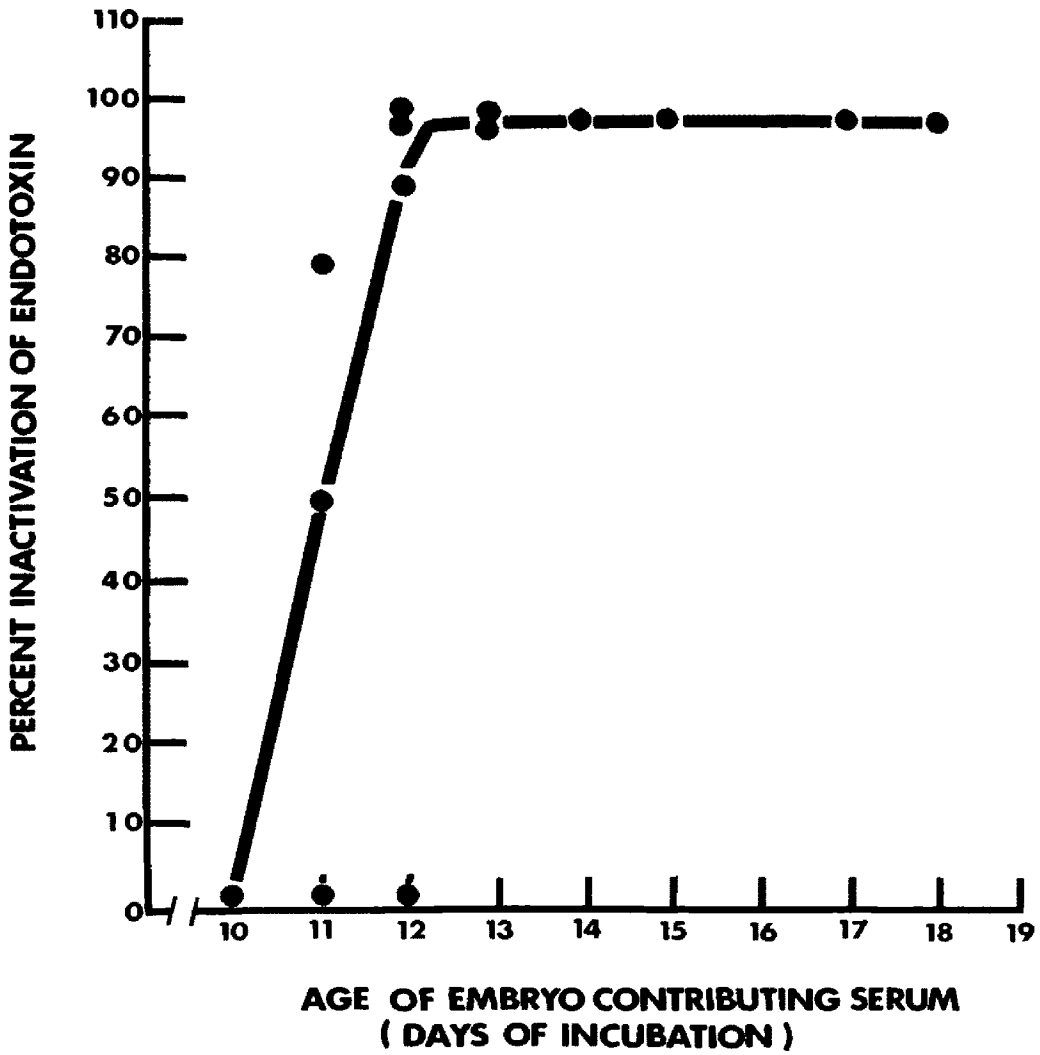

FIG. 11. The capacity of sera from chicken embryos of different ages to inactivate endotoxin. Percent inactivation determined by the Limulus lysate assay. Each point gives the value obtained with sera pooled from embryos of the same age belonging to the same batch of eggs.

TABLE 9. The capacity of sera from chicken embryos of different ages to inactivate endotoxin as determined by bioassay.

Age of embryo contributing serum	$CELD_{50}$[a]	% Inactivation assayed by mouse LD_{50}
Endotoxin control[b]	0.006 ug	0
10	2.6 ug	67
11	ND[c]	> 78[d]
13	ND	> 89[d]
14	5.6 ug	ND
16	ND	87

[a]Chicken embryo lethality assay.

[b]Endotoxin incubated in PBS.

[c]Not done.

[d]LD_{50} higher than the highest concentration of endotoxin-test serum incubation mixture tested.

CHAPTER IV

DISCUSSION

Several hypotheses have been offered for the mechanism of alteration of endotoxin and its subsequent loss of biological properties upon incubation with normal serum. Although some differences in opinion exist, one theory appears to be consistent with all of the manifestations of the endotoxin altering reaction. Rudbach *et al.* (43) have proposed that substances in the blood cleave the intact macromolecular endotoxin complex into smaller subunits, which then complex with a plasma constituent. In this form the subunits are biologically inactive. The importance of complexing with a plasma protein has been demonstrated (36, 56). However, as stated previously, identification by the direct approach of isolation, purification, and characterization of the substance(s) in plasma or serum which alter endotoxin has proven fruitless (42). Therefore an indirect approach was attempted; namely, to test for the capacity of a variety of animal sera to inactivate endotoxin. Hopefully the results of such a study would elucidate the mechanism in serum responsible for the inactivation of endotoxin.

The sera of representative species of several vertebrate classes (*Mammalia*, *Aves*, *Reptilia*, *Amphibia*, and *Osteichthyes*) have been shown to have the capacity to alter endotoxin *in vitro*. Serum from a representative of the class *Chondrichthyes*, the dogfish shark, altered endotoxin only after it has been heated to 56 C for 30 minutes prior to

incubation with endotoxin. Unfortunately, sera from species of the class Cyclostomata were not available for testing. This report adds to the report of Keene et al. (23) in which the plasma of eight mammals were demonstrated to inactivate endotoxin to approximately the same level. There appeared to be no corresponding increase in the capacity of serum to alter endotoxin with the evolutionary development of the vertebrate classes. That is to say, sera from all vertebrate classes tested appeared to have a similar capacity to inactivate endotoxin.

No material tested from invertebrates had the capacity to inactivate endotoxin. The hemolymphs of two invertebrates, the lobster and the crayfish, could not have the capacity to alter endotoxin under experimental conditions identical to those used for the vertebrate sera. Bang (1) has reported that the invertebrate Limulus polyphemus (horseshoe crab) may be killed by infection with a marine, gram-negative bacteria. Also, a heat stable derivative of the marine bacteria and endotoxin from Shigella produced death of the Limulus shortly after injection. Three other invertebrates, the lobster and two other species of crab, were shown to be susceptible to the lethal effects of the toxin obtained from the marine bacteria. It is not known for certain that the lack of a humoral system capable of altering endotoxin in invertebrates influences their susceptibility to the lethal effects of endotoxin. However, Bang (1) has reported that the major pathological consequence of gram-negative infection or endointoxication in Limulus and other invertebrates is a generalized intravascular clotting of the hemolymph; Levin et al. (27) and Reinholt and Fine (35) have reported that lysate of Limulus amebocytes gels in the presence of minute quantities of endotoxin,

whereas endotoxin incubated with human serum no longer causes the clotting of the lysate. It can be hypothesized that if these invertebrates did have a humoral system capable of detoxifying endotoxin similar to the human blood mechanism, the endotoxin might be rendered incapable of causing hemolymph gelation and the extremely lethal effects of endotoxin would be reduced.

There is conflicting data relative to the capacity of the humoral system to alter endotoxin to remain functional after heat-treatment (for examples see 7, 19, 25, 54). The results presented previously generally supported the contention of Cluff (7) and others (19, 54) that alteration of endotoxin by mammalian serum was not diminished by heating the serum to 56 C for 30 minutes. Heated sera from four avian species, also homoiothermic animals, were not changed in their endotoxin altering activity. On the other hand, heat treatment of sera from two poikilothermic species did appear to affect their endotoxin altering capacity. Turtle serum, upon heating, lost its capacity to inactivate endotoxin and heated carp serum was approximately 50% as effective at inactivating endotoxin as unheated carp serum. Quite surprisingly, shark serum appeared to contain an endotoxin altering mechanism which was activated by heating the serum to 56 C for 30 minutes. Conceivably there is a heat-labile inhibitor of the endotoxin altering system in shark serum. However, this hypothesis was never tested.

It would be premature to speculate on the phylogenetic development of a heat stable system coinciding with the change from poikilothermic to homoiothermic animals, but on the basis of the results obtained from heated sera from two poikilothermic animals, such a theory should be

examined. Although the phylogenetic development of a heat stable endotoxin altering system has not been established by this study, the results obtained with shark serum do not negate such an hypothesis. As shown in Fig. 5, the Chondrichthyes departed early from the proposed evolutionary pathway of the homoiothermic animals, and gave rise to no higher forms of animals. Therefore, the apparent heat stability of the endotoxin altering system found in shark serum was not inconsistant with the possibility of the phylogenetic development of a heat stable system.

Stauch and Johnson (54) reported that an incubation temperature of 37 C was optimal for the detoxification of endotoxin by human serum. Although an optimal temperature was not determined, incubation of endotoxin with a variety of animal sera obtained from both homoithermous and poikilothermous animals resulted in greater inactivation at higher temperatures of incubation. Lobster hemolymph was incapable of altering endotoxin at any of these temperatures.

Rosen et al. (41), Landy et al. (25), Skarnes (51) and Skarnes and Chedid (52) have shown that the level of calcium ions greatly affects the capacity of serum to alter endotoxin. Addition of an excess of calcium to all sera which initially were capable of altering endotoxin resulted in a substantial reduction or complete loss of this capacity. Worthy of special mention is the phenomenon exhibited by shark serum. If calcium was added to heated shark serum, no inactivation of endotoxin occurred. It appeared that the mechanism of endotoxin alteration in the serum of the most primitive vertebrate tested, the shark, was similar to the human mechanism, with respect to its inhibition by calcium.

Results of the immunodiffusion assays supported the contention that similar mechanisms of inactivation of endotoxin were operating in a variety of animal sera. In all cases of alteration, regardless of the type of serum used, immunodiffusion patterns revealed the loss of the major, slowly migrating antigen of endotoxin. Concomitantly with this was the formation of a faster migrating antigen. As the rate of migration of an antigen through a gel matrix is dependent, for the most part, on the molecular size of the antigen (9), these results may be intrepreted as evidence that the endotoxin complex is cleaved into smaller subunits when exposed to these various animal sera. If so, these results appeared to be in concert with the contention of Rudbach et al. (43) that the endotoxin complex was dissociated when exposed to serum.

Turning now to the results of experiments designed to test for the ontogenetic development of the mechanism responsible for alteration of endotoxin, several different types of assays revealed that there was a slight increase in the endotoxin altering activity of serum as the chicken embryo matured from 10 to 13 days of age. An increase in resistance to the lethal effects of injection of endotoxin has been reported to occur in this general interval of maturation (15, 16) and shown more specifically to occur between the 11th and 13th day of incubation (Fig. 9). It might be hypothesized that the humoral mechanism responsible for the inactivation of endotoxin in vitro may have some role in the resistance of chicken embryos to the lethal effects of endotoxin in vivo. However, the change in the in vitro capacity of serum to inactivate endotoxin did not appear to account for the magnitude of change in susceptibility to injection of endotoxin in vivo. Factors,

other than the development of a humoral detoxifying system, probably influence the *in vivo* resistance of chicken embryos to endotoxin. The reticulo-endothelial system has been implicated repeatedly in the removal of circulating endotoxin (6, 8) and in its subsequent detoxification by macrophages (13, 48). Interestingly, Romanoff (39) has reported that monocytes are not detectable in the developing chicken embryo until, at the earliest, the 14th day of incubation. Finkelstein and Ramm (16) have postulated that the conversion of a mesonephric to metanephric kidney in the developing embryo, a process which was completed near day 15, affects the susceptibility of the chicken embryo to endotoxin. Finkelstein (15) found that the level of blood sugar was greatly reduced in 11-day old embryos following injection of endotoxin, but older embryos had only slight changes in blood sugar levels after exposure to endotoxin.

According to Rudbach *et al.* (43) inactivation of endotoxin by serum requires both surface active agents, possibly bile salts, and a serum protein which binds to the dissociated subunits of endotoxin. The sera from embryonic chickens, which were shown to possess the capacity to inactivate endotoxin, should contain these substances in order to be in harmony with this hypothesis. Bile salts have been reported to be in circulation by the seventh day of incubation (24). Marshall and Deutsch (28) have reported that all of the major electrophoretic components of adult serum, also are present in the serum of 10-day old embryos.

The evolutionary origin of the endotoxin altering mechanism appeared to be found in the transition zone between invertebrates and

vertebrates. Comparison of the hemolymph of the invertebrates, shown to be deficient in endotoxin altering activity, to serum with the capacity to alter endotoxin, with hopes of further elucidating the nature of the endotoxin altering mechanism, was not possible; the composition of the hemolymphs tested have not been reported in sufficient detail to allow for such a comparison (11). The sera of all the vertebrates shown to possess endotoxin altering activity contained $alpha_1$-globulin, $alpha_2$-globulin, and a component with a similar electrophoretic mobility as human $beta_1$-lipoprotein (11), all of which have been implicated in the mechanism of inactivation of endotoxin (49, 50, 59). The components of complement which have been indicated as possibly important constituents in the inactivation of endotoxin (5, 21, 42) were also present in the sera of all vertebrates tested (17). It can be hypothesized that, as a result of the large number and diversity of sera tested, specific antibody against the O antigen of endotoxin is probably not necessary for inactivation.

Certain aspects of this study have shown that the mechanism of inactivation of endotoxin was similar in the sera of many animals to the mechanism found in human serum. This observation removed the limitation of examining only human and other mammalian sera in order to investigate the mechanism responsible for inactivation of endotoxin.

On the basis of the experimental data reported herein, most of the objectives of this study, outlined previously, have been attained. Sera from various species of animals and from chicken embryos of varying ages were assayed for capacity to alter endotoxin. The results showed that the mechanisms of the altering reactions were not inconsistent with that

proposed for alteration of endotoxin by human serum. Serum samples were obtained from species which could and species which could not alter endotoxin, and the attainment of this capacity appeared to have occurred during the evolution of the vertebrates from invertebrates. A less complex system for subsequent studies on the nature of the endotoxin altering mechanism was discovered. Shark serum contained a heat-stable, calcium inhibitable, endotoxin altering system, the manifestations of which are indistinguishable from that in human serum. Inasmuch as the variety of proteins in shark serum is lesser than that found in human serum (34), the former would appear to present a valuable model for subsequent investigations on the nature of the endotoxin altering factor(s).

CHAPTER V

SUMMARY

Normal sera from animals located at different phylogenetic levels were tested for the capacity to inactivate endotoxin. It was found that sera from species belonging to 6 classes of vertebrates: Mammalia, Aves, Reptilia, Amphibia, Osteichthyes, and Chondrichthyes have a capacity to inactivate endotoxin. On the other hand, hemolymphs from two invertebrate species, the lobster and crayfish, were found to be incapable of inactivating endotoxin. It was postulated that the phylogenetic origin of the endotoxin altering mechanism is to be found in the transition from invertebrates to vertebrates.

In general, the mechanism of inactivation of endotoxin by sera from these various vertebrate classes was shown to be similar to that employed by human serum. This mechanism was found to be influenced by the temperature of incubation and inhibited by excess amounts of calcium. Also, an analogous mechanism for the inactivation of endotoxin by sera from a variety of animals was indicated by immunodiffusion analysis.

Sera from chicken embryos of different ages, 10 to 18 days old, also were shown to have the capacity to inactivate endotoxin. The slight increase in the capacity of serum from the older chicken embryos to inactivate endotoxin, as determined by *in vitro* assay, could be correlated with the increased resistance of the older chicken embryos to the lethal effects of endotoxin *in vivo*.

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